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The amphibian micronucleus test, a model for in vivo monitoring of genotoxic aquatic pollution

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This review deals with the experiments carried out over the last decade on the detection of the genotoxicity in aquatic medium (fresh water) using amphibian larvae. Three amphibian species have been widely used: two species of urodeles, the Spanish newt or pleurodele (*Pleurodeles waltl*) and the Mexican axolotl (*Ambystoma mexicanum*), as well as one anuran, the South African clawed frog or common platanna (*Xenopus laevis*). The protocol carried out on these amphibians, at the Developmental Biology Center in Toulouse, France, allows the direct detection of genotoxic agents in the rearing medium of the animals by measuring the induction level of micronucleated erythrocytes of larvae exposed to pure substances, physical agents, complex mixtures of substances like drinking and surface water, domestic and industrial wastes, or any aqueous effluent.

After introducing the test procedure and the rearing conditions of the animals, the results obtained with the amphibian micronucleus assay are presented with respect to the chronological order of the different steps leading to the publication of the standardized method in 1992 and to the state of the art in the field of in vivo eco-genotoxicology of aquatic media, where amphibians, thanks to the works reported in this paper, play a major role.

Then, the choice of future development areas in the field is discussed in terms of validity of the use of amphibians as genotoxicity bioindicators in the aquatic environment, considering the many advantages of this model and its complementarity to the commonly used in vitro test-systems.

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INTRODUCTION

Increased environmental pollution can be attributed to a variety of factors resulting from new industrial and agricultural technologies together with changes in our way of life. Moreover, the nature of the pollution itself has become more diverse. Whatever the origin of the pollution, it tends to find its way into the aquatic environment. Genotoxic pollutants affect the aquatic ecosystem, and their presence in the water can also have repercussions on non-aquatic species via food chains or simply from drinking the water. One should therefore be aware of the hidden risks stemming from potential genotoxic substances in the aquatic environment. Moreover, a considerable time may elapse between the action of the mutagenic agent and the outward signs of its effects. The relationship between cause and effect may thus become obscured.

The mutagenic risk is particularly apparent in prokaryotes, and readily discernible in plants and animals with a rapid rate of reproduction, although it is often not very perceptible in plants and animals (including humans) with a slower reproduction rate. It should be remembered that the manufacture and use of aggressive mutagenic substances is too recent to allow judgment of effects over a relevant number of generations. The mutagenicity of an unknown substance is usually evaluated by putting it in contact with a living system, which is then examined for genetic damage. It is generally agreed that it is difficult to extrapolate results obtained in one living system to another one, or even from animal to man. Nevertheless, the commonly used tests, as a first step, are based on bacteria, such as the Ames test (AMES et al., 1975; MARON & AMES, 1983). The main advantages are that such tests can be carried out rapidly and are low in cost. One of the main drawbacks of these bacterial tests for the detection of genotoxic substances in water is that they are relatively insensitive, and in general they cannot be used on unconcentrated water samples (WEAVER et al., 1981).

Ideally, one should evaluate the biological hazards of environmental genotoxic pollutants in situ. In vivo mutagenicity tests applied to unconcentrated water samples represent a step in this direction (CHOUROULINKOV & JAYLET, 1989; JAYLET & ZOLL, 1990). They give an indication of the overall genotoxic potential of the water under testing. One example is the micronucleus test adapted to larvae of amphibians that we developed over the last decade. The larvae can be reared not only in containers filled with unconcentrated water samples (laboratory conditions), but also in running water of various sources (effluents of factories, river water or even drinking water).

In amphibian larvae, as in most eukaryotes, chromosome and genome mutations result in the formation of micronuclei (fig. 1). These micronuclei are small intracytoplasmic masses of chromatin resembling small nuclei (fig. 1A). They are formed from chromosome fragments or complete chromosomes which have not migrated to a spindle pole during anaphase (fig. 1C). Therefore, the formation of micronuclei stems from either chromosome fragmentation or a malfunction of the mitotic apparatus. In the former case, micronuclei correspond to chromosome fragments that, having lost the centromere, have been unable to connect with the spindle fibers. In the latter case, they arise from complete chromosomes lagging at anaphase due to spindle abnormalities. Clastogenic compounds and spindle poisons both lead to an increase in the number of micronucleated cells.
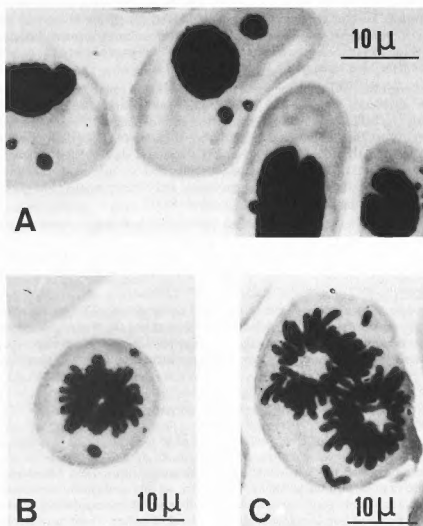
Fig. 1. – Aspect of the micronucleated erythrocytes of pleurodele larvae during (B, C) and after (A) cell division. Larvae have been treated during 12 days with BaP (0.5 ppm) and blood smears were fixed in methanol (5 minutes), stained with Masson's acid hemalun (12 minutes) and then placed under running water for 10 minutes.

A. – Four micronucleated erythrocytes (interphase). After treatment with a strong genotoxic agent, it is not rare to observe cells containing 4 or more micronuclei (cell on the right), whereas in the control larvae, most of the micronucleated cells contain only one micronucleus but rarely two or three.

B. – Erythrocyte of pleurodele during metaphase, including 3 chromosomal fragments.

C. – Erythrocyte of pleurodele during anaphase, in which a chromosomal fragment and probably a whole chromosome are not incorporated to the spindle.

EVANS et al. (1959) were the first to suggest counting cells containing micronuclei as a method for the evaluation of cytogenetic damage. Since then, induction of micronuclei has been widely used for genotoxicity testing. A detailed description of the micronucleus test using bone marrow polychromatic erythrocytes from small mammals is given by SCHMID (1976). Results from the micronucleus test and recommendations for its practical application have been reviewed by HEDDLE et al. (1983).

In the animal kingdom, micronuclei formation has been studied mainly in mammals. In aquatic vertebrates, micronucleus tests using fish have been carried out on different species: the mudminnow (*Umbra limi*) and the brown bullhead (*Ictalurus nebulosus*) (METCALFE, 1988), the Eastern mudminnow (*Umbra pygmaea*) (HOOFTMAN & DE RAAT, 1982), the white croaker (*Genyonemus lineatus*) (CARRASCO et al., 1990) and *Heteropneustes fossilis* (DAS & NANDA, 1986). In our hands, a similar test, on red blood cells from three other fish species (*Brachydanio rerio*, *Cyprinus carpio* and *Nothobranchius rachowi*), did not lead to statistically significant results (unpublished data).

In 1987, KRAUTER et al. demonstrated micronuclei formation in peripheral erythrocytes of *Rana catesbeiana* tadpoles after irradiation. They proposed this animal for in vivo genotoxicity studies. Other amphibian species have been designed to score the level of micronucleated cells in animals exposed to genotoxic substances in water and to monitor aquatic genotoxic pollutions in the environment.

This paper has two major purposes: to present the procedure of the micronucleus test applied to 3 amphibian larvae, the pleurodele, the axolotl and the platanna; and to review the works done until now on the use of these three amphibian species to evaluate the genotoxic impact of chemicals or physical agents and to detect genotoxicity in aquatic media.

MATERIALS AND METHODS

THE AMPHIBIAN MICRONUCLEUS TEST

The test procedure has been established in two species of urodeles, *Pleurodeles waltl* (the Spanish newt or pleurodele; family Salamandridae) and *Ambystoma mexicanum* (the Mexican axolotl; family Ambystomatidae), as well as in the anuran *Xenopus laevis* (the South African clawed frog or common platanna; family Pipidae). These three amphibians are abundant egg-layers: a female of pleurodele or axolotl can lay up to 1000 eggs at once, while 2000 or 3000 is not rare in platannas. Their rearing and development are now well described. The details of the test procedure have been described in the publications mentioned above, and for urodeles documentation sheets have been published by the French Standards Institute (ANONYMOUS, 1987, 1992).

Rearing conditions

Urodeles

Similar methods are used to rear both axolotl and pleurodele larvae. After they are laid, the eggs are placed in an aquarium. The water is normal tap water filtered through

active carbon or "ultrapure" water reconstituted with salts. The young larvae eat only live food. After hatching, the animals are fed on freshly hatched artemia (*Artemia salina*) or daphnia (any species). Then the food is switched to *Chironomus* larvae. Usually this latter food is subsequently used throughout the experiments. The temperature of the water can range from 12 to 20°C. Within this range, an increase in temperature increases the growth rate of the animals. It is therefore possible to change the rate of development of animals depending on requirements, and several groups at different stages of development can be studied at the same time. However, the temperature dependence of the mitotic index must be taken into account. All treatments are carried out at 20°C. They are always carried out after a 8-day habituation period at this temperature.

Platanna

Xenopus tadpoles are fed on dehydrated aquarium fish food. The temperature of the water can range from 18 to 23°C; the growth rate also depends on the rearing temperature. All treatments are carried out at 22°C after a 6-day habituation period at this temperature.

Treatment stage

The size of the larvae must be large enough to allow an easy taking of blood samples. They must also be at a stage of intense erythropoiesis with a large number of divisions of the red blood cells in circulating blood. As the rate of growth is a function of temperature, and as treatment should be carried out when the larvae are in an identical physiological state, age cannot be used as a reference. An accurate morphological marker is required. For the axolotl, we have found that treatment must start when the hind limb buds of the larvae exhibit slight indentation (onset of formation of the two first digits). For pleurodele, treatment is started when the hind limbs present four well formed digits with an outline of the fifth (fig. 2A). In both cases, the mean size of the larvae is around 30 mm (about 6 weeks after laying). They reach 40 mm within the next 10 days. The growth of *Xenopus* larvae is quicker and the test can begin 2 weeks after laying. At this time, one must choose larvae at stage 50 (fig. 2B) of the chronological table of NIEUWKOOP & FABER (1956) (hind limb bud longer than broad, constricted at base).

Experimental design

The treatment procedure is basically the same for all three species. They are treated in groups of twenty in 5-liter flasks filled with 2 liters of the sample (100 ml of water per larva). Control groups are reared in purified water. The media are renewed and food is added every 24 hours. At the end of the treatment period (generally 12 days for pleurodele and platanna and 10 days for axolotl), the animals are anesthetized with 0.02 % tricaine methane sulfonate. Blood samples are taken by cardiac puncture into heparinized micropipettes (20 % solution at 5000 IU/ml). Blood smears are made, then fixed in methanol and stained with hemalun solution. Slides are examined under the microscope with an immersion lens ($\times$ 100). For each animal, the number of micronucleated red blood cells per 1000 cells is determined (fig. 2C-D). In these conditions, the frequencies of micronucleated erythrocytes in the control animals is about 0.5 − 1 % for urodeles and 0.1 % for platanna.

Significance of the test

For each group of animals, the results (level of micronucleated red cells per 1000) obtained for the individual larvae are arranged in increasing order of magnitude and the medians and quartiles calculated (see example in Table III). In most studies, the statistical method used to compare the medians is based on the recommendations of MAC GILL et al. (1978). It consists in determining the theoretical medians of samples of size N (where $N \geqslant 7$) and their 95 % confidence limits expressed by $M \pm 1.57 \times IQR / N^{-1}$, where M = median and IQR = Inter Quartile Range (see example in fig. 3). In these conditions, the difference between the theoretical medians of the test groups and the theoretical median of the control group is significant to within 95 % certainty if there is no overlap. The result is then positive. In the absence of statistical calculations, a result is considered positive if the two following conditions are satisfied : (1) the median of the treated animals is twice that of controls; (2) the lowest quartile of the treated animals is above the highest quartile of the controls. A positive result can be taken for any duration of treatment, but in general 4 to 8 days is long enough for a strongly clastogenic substance used at maximal concentration (MC). MC is defined as half the lethal concentration within a period of 6 days.

BACTERIAL TEST-SYSTEMS

This paper relates the results obtained in comparative studies including in vitro test systems on bacteria: the Ames test, detecting point mutation on *Salmonella typhimurium*, the fluctuation test, which is a modification of the Ames test where the compounds under study are exposed to bacteria in a liquid medium instead of the agar plates used in the Ames assay, and the SOS chromotest, showing primary DNA damage on *Escherichia coli*.

Fig. 2. – Amphibian larvae used for the micronucleus assay and corresponding erythrocytes showing micronucleated cells.

    A. – Pleurodele larva at the stage of treatment, i.e. stage 53b, according to the chronological development table of GALLIEN & DUROCHER (1957), or stage 45-46 of the development table of SHI & BOUCAUT (1995). At this stage of development, the pleurodele larva is a small aquatic animal (about 35 mm long). Axolotl larva has the same appearance and the same size as pleurodele larva at the treatment stage.

    B. – *Xenopus* larvae at the stage of treatment. Left, larva at stage 50 of the chronological table of development of NIEUWKOOP & FABER (1956), corresponding to the beginning of the test. Right, larva at stage 54, 12 days later, corresponding to the end of the test. During the testing period, at 22°C, the animals are growing very quickly, increasing in size from 22-26 mm (st. 50) to 58-65 mm (st. 54).

    C. – Blood smear from a pleurodele larva, stained with Masson's hemalun, showing red blood cells with their main nucleus and one micronucleated cell. Pleurodele and axolotl erythrocytes are big ovoid cells (about 30 µm in diameter).

    D. – Blood smear from a *Xenopus* larva, stained with hematoxylin, showing one micronucleated erythrocyte. *Xenopus* erythrocytes are smaller than those of urodele species (about 10 µm in diameter).
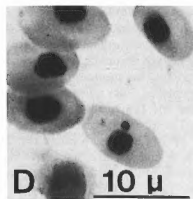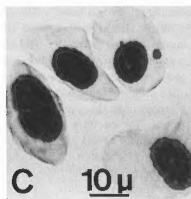
Principles of the tests

Ames test

The most widely used and validated bacterial reverse-mutation test is that devised by AMES et al. (1975). The Ames test (also called the *Salmonella*-mammalian microsome test) employs strains of *Salmonella typhimurium*, which are unable to produce their own histidine. Each of these strains bears a specific defect or mutation in the metabolic pathway to histidine. Reverse mutations restore the ability to synthesize histidine. These reverse mutations are induced by specific mutagens. Mutagens that cause base pair substitutions can be distinguished from those that lead to frameshift mutations by the use of different strains. If the mutant strain (his-), which is unable to grow in media devoid of histidine, regains the ability to synthesize histidine after being exposed to an extract, then the extract is assumed to contain a mutagen.

However, unlike mammals, bacteria lack the necessary oxidative enzyme systems that metabolize foreign compounds to electrophilic compounds that interact with DNA (indirect mutagens). Bacterial tests are thus carried out in the presence of a liver microsomal fraction that contains such enzymes. This is usually prepared by ultracentrifugation of a cell homogenate of rat liver. The metabolic activity of this fraction (S9 fraction that contains cytochrome P 450 / P 448 oxidases) is enhanced by prior treatment of the rats for several days with an inducer (usually Aroclor 1254). The so-called S9-mix is made up from this S9 fraction that is buffered and supplemented with NADP and glucose-6-phosphate. A standard procedure has been drawn up for this test. Each test is carried out several times, both with (detection of indirect mutagens) and without metabolic activation (detection of direct mutagens).

Fluctuation test

GREEN et al. (1976) have developed another mutagenicity test based on the Ames test that is both simple and sensitive. The same bacterial strains are exposed to the test substances in a liquid medium. The auxotrophic mutant strains (around 3.10^7 bacteria) are placed in a culture medium containing glucose, a small quantity of histidine and the test substance. Activation medium (S9) may be added later. An overall volume of 5 ml is generally employed. Fifty independent cultures in 0.1 ml containing around 5.10^5 bacteria are then set up. The small bacterial population minimizes the number of preexisting revertants. The tubes are incubated at 37°C overnight. The bacteria are allowed to grow until the supply of histidine is exhausted. This is referred to as the period of auxotrophic growth. The next day, 2 ml of a selective medium (containing glucose, a pH indicator such as bromocresol, but without histidine) is added to each culture. The tubes are incubated for a further 2 or 3 days at 37°C. Only the revertants (prototrophic for histidine) are able to grow. This leads to a fall in pH, and the tubes turn from blue to yellow. This is referred to as the phase of prototrophic growth. The number of positive tubes are counted (prototrophic growth = yellow) in a batch of 50 tubes. Using appropriate statistical methods, the number of positive tubes are compared between the tubes containing test substances and the control tubes. The test is replicated a number of times, and similar controls to those used in the classic Ames test are run in parallel.

SOS chromotest

The genotoxic effects observed in the bacterial tests are often not direct actions of the agent. They are often the outward signs of the overall responses of the cell to this action. In *Escherichia coli*, DNA-damaging treatments can activate a set of functions known as the SOS responses. This has been exploited by an operon fusion placing lac Z, the structural gene for ß-galactosidase, under control of the sfiA gene, a SOS function involved in the inhibition of cell division. A simple and direct colorimetric assay of this SOS response to DNA damage has been developed, called the SOS chromotest (QUILLARDET et al., 1982; QUILLARDET & HOFNUNG, 1985). Mutagenicity is evaluated quantitatively in terms of the SOS-inducing potency. Enzyme activity after incubating the tester strain in the presence of various amounts of the test compound is measured colorimetrically. Aliquots of a dilution of exponential phase cultures are placed in glass test tubes containing the compound to be tested. After 2 hours incubation at 37°C with shaking, ß-galactosidase and alkaline phosphatase activities are assayed.

The classic microsomal activation preparation may also be added to the incubation mixture. To correct for the inhibition of protein synthesis that may be induced by certain substances, the strain is made constitutive for synthesis of alkaline phosphatase. This enzyme is non-inducible by DNA-damaging agents. The ratio of the two activities (ß-galactosidase to alkaline phosphatase) is taken as a measure of the specific activity of ß-galactosidase.

RESULTS OBTAINED WITH THE AMPHIBIAN MICRONUCLEUS TEST

GENOTOXICITY OF X-RAYS AND CHEMICALS

Initially, the sensitivity and dose-response of the micronucleus test were evaluated with X-ray irradiations as well as known physical clastogenic agents and chemicals exposure, in pleurodele larvae.

SIBOULET et al. (1984) measured the frequences of micronucleated red blood cells in the animals 6 days after X-ray irradiation. A dose of 6 rad (relatively weak) leads to a significant effect. The dose-response is approximately linear up to 150 rad, after which the slope falls and the maximal effect is reached at 600 rad.

Two years later, JAYLET et al. (1986a) determined the most suitable larval stage for testing chemicals using pleurodele larvae reared in water containing one of the 4 following compounds: benzo(a)pyrene (BaP), ethyl methanesulphonate (EMS), diethyl sulphate (DES) and N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG). Response curves as a function of treatment duration over a period of 16 days were plotted for 3 different concentrations of the 4 compounds in order to optimize conditions for a low dose micronucleus test.

The same year, GRINFELD et al. (1986) focused on BaP studies. These authors exposed pleurodele larvae to different concentrations of the substance for various lengths of time. Frequencies of micronuclei in circulating erythrocytes were determined at different times after termination of the treatment. The incidence of micronuclei in larvae kept for 8 days

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Table I. - Summary of the results obtained for different chemical classes and X-rays
     with *Pleurodeles waltl* (from FERNANDEZ et al., 1993).

Chemical class	Number of chemicals tested	Positive responses	Negative responses
Miscellaneous	24	9	15
Amines (aromatic, aliphatic)	7	5	2
Nitroso compounds	5	5	0
Polycyclic carbocycles	5	5	0
N-, S-, O-mustards	2	2	0
Aziridines	1	1	0
Carbamates	1	1	0
Oxygenated sulfur	2	2	0
Total	47	30	17
X-Rays	12 doses	12	0

in BaP containing water displayed a marked increase with dose up to 0.075 ppm and a
more gradual one with higher doses, reaching 158 per 1000 at 0.75 ppm. The lowest dose
at which a significant increase could be discerned was 0.01 ppm. Uptake and release was
studied with tritiated BaP. Larvae concentrated BaP rapidly, attaining maximal levels after
12 hours. The ratio of radioactivity in larvae to that in an equivalent volume of
surrounding water was about 200, independent of the amount of BaP added. The marked
bioaccumulation potential of newt larvae partially explains why it is not necessary to
concentrate mutagenic micropollutants in samples of natural or drinking water to detect
genotoxic effects

   Results with 19 organic compounds have been published by FERNANDEZ et al. (1989).
Most of them were known or suspected mutagenic or carcinogenic substances in mammals.
The results were compared with published data from other tests used to detect the
clastogenic or mutagenic properties of chemicals.

   In 1993, FERNANDEZ et al. published results obtained with 47 different chemical
compounds, after 12 days or/and 8 days of exposure in pleurodele, axolotl and *Xenopus*
larvae. The overall results obtained for the different chemical classes tested with pleurodele
are shown in Table I. For comparative purposes, literature data have been collected on
other short-term genotoxicity tests and on long-term carcinogenicity assays in rodents
(Table II). In this study, the newt micronucleus assay on pleurodele larvae was found to
agree better with the Ames test (bacterial test) (percent concordance with the newt
micronucleus test: 86 %) than with the rodent micronucleus test (71 %).

Using pleurodele larvae, the genotoxicity of 7 polycyclic aromatic hydrocarbons (PAHs) was compared and the influence of UVA irradiation evaluated by FERNANDEZ & L'HARIDON (1992). The authors classified the PAHs in the following order of genotoxicity: benz(a)anthracene (BA) = 7,12-benz(a)anthraquinone > 7,12 dimethyl-benz(a)anthracene (DMBA) > 9,10-dimethylanthracene; whereas anthracene, 9,10 anthraquinone and dibenz(a,h)anthracene were not found to be clastogenic. Under lighting conditions (UVA irradiations for 24 hours) of the rearing media alone (water containing the tested substance), and then tested on larvae in the dark, BA (50 and 100 ppm) gave rise to clastogenic products, whereas DMBA (12.5; 25 and 50 ppb) gave no positive response in the test.

More recently, in the same area of research, DJOMO et al. (1995) studied the genotoxicity of 4 polycyclic hydrocarbons representing a part of the major fraction of hydrocarbons found in a crude oil. The authors confirmed the strong genotoxic potential of benzo(a)pyrene, whereas the genotoxicity of naphtalene was weak. In contrast, the two other compounds tested, anthracene and phenanthrene, gave negative responses in the newt micronucleus test.

L'HARIDON et al. (1993) applied the same test system to evaluate the genotoxicity of amines and/or potential nitrosating agents (nitrite and nitrate) in vivo. The authors obtained negative results under varying rearing conditions (lighting conditions, pH 8-6-5) with atrazine, diethanolamine alone or in combination with sodium nitrite or nitrate. However, the genotoxicity of N-nitrosoatrazine (NAT) at 7.5 and 15 ppm and N-nitrosodiethanolamine (NDELA) at 12.5-25 and 50 ppm, was demonstrated. In conclusion, the authors suggested that, at the concentrations used (close to those which may be encountered in a polluted natural aquatic environment), if NAT or NDELA are formed, the amounts produced are probably too low to yield a positive response in the newt micronucleus test.

The cytogenetic effects of mercury compounds have been widely studied in plants, *Drosophila* and tissue culture cells, but to our knowledge they have not been evaluated in vertebrates in vivo. Pleurodele larvae were raised in water containing low concentrations of methyl mercuric chloride or mercuric chloride (ZOLL et al., 1988). It should be noted that a low concentration of the two substances (12 ppb) gave a positive result and that equivalent concentrations in the water of both mercuric compounds led to similar levels of micronucleated cells. The test gives positive results for concentrations below those often found in samples of contaminated water (GIRAUD & GUILLET, 1972). Both chromosome aberrations and abnormalities in cell division were observed in cells from animals treated with these two substances. Bioaccumulation of both compounds was also evaluated by determination of mercury levels in the larvae. After 12 days of treatment, concentration factors (concentration in the organism / concentration in the water) of 1200 and 600 were found for methyl mercuric chloride and mercuric chloride respectively.

In order to investigate the generality of the micronucleus test in pleurodele, larvae from another urodele, the axolotl, were reared in water containing either of the compounds benzo(a)pyrene (BaP) or ethyl methane sulfonate (EMS) (JAYLET et al., 1986b) The level of micronucleated erythrocytes on blood smears was compared with control samples from larvae reared in fresh water. The optimum larval stage for this test system was determined. The effects of the indirect mutagen (BaP) and the direct mutagen

(EMS) were found to depend on both dose and exposure to the clastogen. For BaP, positive results were obtained after 8 days of treatment at a concentration of 0.025 ppm. After 10 days of treatment at a concentration of 0.1 ppm, numerous micronuclei were seen (< 25 %). Positive results were also obtained with EMS after 8 days of treatment at a concentration of 24 ppm. At 62 ppm, positive results were found after 6 days, while at 124 ppm positive results were found after only 4 days. The results with both these agents show that the axolotl also holds promise as an in vivo test system for the detection of low concentrations of clastogens in the aquatic environment. This is not very surprising, since the axolotl is morphologically and biologically rather similar to the pleurodele at both the embryonic and larval stages, although not of the same family.

The third species used was *Xenopus laevis*. It differs in a number of respects from the previous two species, including its feeding behavior. Three different variables, temperature, stage of larval development and frequency of renewal of the test substance, were investigated using EMS as the clastogenic compound. In addition, a dose-response curve was established for BaP in order to determine the limits of sensitivity of the test (VAN HUMMELEN et al., 1989). The lowest concentration (0.03 ppm) gave a negative response. From 0.06 ppm up to 0.5 ppm, an approximately linear increase in median value of cells with micronuclei was observed. This linear response indicates that the test is

Table II. - Comparison of qualitative results obtained for different chemical substances and X-rays in the newt *Pleurodeles waltl*, with various short-term genotoxicity and long-term rodent carcinogenicity tests (from FERNANDEZ et al., 1993). Abbreviations for chemical substances: AO, acridine orange; Aro, Aroclor 1254; AT, atrazine; BaP, benzo(a)pyrene; BrFo, bromoform; BHA, butylated hydroxyanisole; CAP, ε-caprolactam; ClFo, chloroform; CP, cyclophosphamide monohydrate; DEIA, diethanolamine; DES, diethyl sulfate; DMSO, dimethyl sulfoxide; ECH, epichlorhydrin; EtOH, ethanol; EB, ethidium bromide; EMS, ethyl methane sulfonate; DBE, ethylene dibromide; ENNG, N-ethyl-N'-nitro-N-nitrosoguanidine; ENU, N-ethyl-N-nitrosourea; GSH, glutathione; HEMPA, hexamethylphosphoramide; InM, indomethacin; MeC, mercuric chloride; 3-MC, 3-methylcholanthrene; MMeC, methyl mercuric chloride; MCA, monochloramine; NAT, N-nitrosoatrazine; NDEIA, N-nitrosodiethanolamine; PB, phenobarbital; Py, pyrene; NaF, sodium fluoride; NaOCl, sodium hypochlorite; NaNO₂, sodium nitrite; NaNO₃, sodium nitrate; NaNO₂, sodium nitrite; STS, sodium thiosulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate; o-TOL, o-toluidine; TCAH, trichloroacetaldehyde hydrate; BA, benz(a)anthracene; CAF, caffeine; CAN, Captan; CARB, Carbaryl; COL, colchicine; DMBA, 7,12-dimethylbenz(a)anthracene; ETI, ethyleneimine; FA, formaldehyde; N-CARB, N-nitrosocarbaryl. Abbreviations for results: +, positive; (+), weakly positive; -, negative; ?, inconclusive; L.P, limited positive; L.N, limited negative; E.E, equivocal evidence; +/-, different results depending on the authors or on the cell types; A, aneuploidy; P, polyploidy.

		Newt MN test	Carcinome in rodents	Ames test	C.A.	Rodents MN test
1	AO	+	+	+	+	+
2	Aro	-	+	-	-	-
3	AT	-		-	-	-
4	BaP	+	+	+	+	+
5	BrFo	+		+		
6	BHA	-	L.P	-	-	-
7	CAP	(+)	-	-	+/P (Hum)	-
8	ClFo	-	+	-	-	
9	CP	+	+	+	+	+
10	DEIA	-				
11	DES	+	+	+	+	+
12	DMSO	-		-	-	
13	ECH	+	+	+	+	-
14	EtOH	+	-	-	-	
15	EB	+		+		
16	EMS	+	+	+	+	+
17	DBE	+	+	+	+	-
18	ENNG	+	+	+	+	
19	ENU	+	+	+	+	
20	GSH	-		-		
21	HEMPA	+	+		+	+
22	InM	-				
23	MeC	+		-/+	-/+/A	
24	3-MC	+	+	+	+	+
25	MMeC	+		-/+	+/A	
26	MCA	+		+		
27	NAT	+			+	
28	NDEIA	+	+	+	+	
29	PB	-	?/ (Hum:-)[a]	+	+	-
30	Py	+		+/-	-	-
31	NaF	-	E.E	-	-/+	-
32	NaOCl	+		+	+	-
33	NaNO₃	-	?		+	-
34	NaNO₂	-	?	+/-	+	-
35	STS	-		-	-	
36	TPA	-	?	-	+/-/P/A	-
37	o-TOL	+	+	+	+	-
38	TCAH	(+)	+	+	-	
39	BA	+	L.P	+		
40	CAF	+	L.N	-	+	-
41	CAN	(+)	L.P	+	+	+
42	CARB	+		+	+/A/P	-
43	COL	-	-	-	-	+
44	DMBA	+	+	+	+	+
45	ETI	+	+	+	+	+
46	FA	-	+	+	+	+
47	N CARB	+	+	+	+	-
48	X-rays	+	+	+	+	+
	%[b]	100%	76%	86%	83%	71%

a. From UPTON et al. (1984).

b. Percent concordance with newt micronucleus test.

reliable, although the lower frequencies of cells with micronuclei at doses above 0.5 ppm are probably accounted for by a lower rate of growth of the larvae exposed to high doses of BaP In fact, above BaP concentrations of 1 ppm the larvae eat less and grow more slowly than the larvae in the other samples. The mitotic index is thus lower and, since the production of micronuclei depends on cell division, a fall in the mitotic index results in a decrease in the number of red blood cells with micronuclei. It is worth noting that a similar phenomenon occurs for the same concentrations in axolotl and pleurodele with BaP.

All these results demonstrate the sensitivity and the reliability of the test for known genotoxic agents experimentally added to the rearing water It was important to find out whether the amphibian micronucleus test was also applicable to real-world situations.

GENOTOXICITY IN DRINKING WATER

Using pleurodele larvae, JAYLET et al. (1986c, 1987) evaluated the mutagenic activity in drinking water taken directly from the tap supplying the laboratory. Groups of larvae were reared in tap water, while control animals were reared in tap water filtered over sand and active carbon, to remove micropollutants. Seven separate tests carried out in samples of tap water taken at different times throughout the year gave positive results of varying degree depending on the time of the year (see results in Table III and fig. 3). The authors concluded that this test was therefore able to detect clastogens in normal drinking water and that it could be used for quality control of drinking water during the various stages in the treatment of raw water. The aim of these studies was to determine whether the test was sufficiently sensitive to be used directly on drinking water samples without prior extraction or concentration of the micropollutants. To our knowledge, this was the first description of a test system using an aquatic vertebrate for the detection of potential clastogens in samples of tap water.

To explain the positive results obtained in these previous experiments, various hypothesis have been formulated. Genotoxic micropollutants in drinking water can come from a variety of origins. Various possibilities suggest themselves which are not mutually exclusive. The micropollutants may be (1) residual chlorine from the chlorination processes used for water disinfection, (2) substances produced by the action of chlorine on organic matter forming halogenated organic compounds, or (3) even substances present in the raw water.

(1) In order to examine the first possibility, further experiments were carried out.

GAUTHIER et al. (1989) evaluated mutagenic activity of chlorinated and monochloraminated water devoid of all organic matter on pleurodele larvae. The level of micronuclei in erythrocytes was compared between a group of larvae reared for 12 days in chlorinated reconstituted ultrapure water treated with sodium hypochlorite and a control group reared in the reconstituted water Sodium hypochlorite was added when both the food and medium were changed each day. Chlorine levels of 0.125 and 0.25 ppm led to significant elevations of micronuclei. The possibility of indirect effects of chlorine through chemical interactions with the food were also investigated, using the following scheme: larvae were left for 3 hours in chlorinated reconstituted ultrapure water and then placed in non-chlorinated water. Food was only introduced when they were transferred to

Table III. - Results of tests on laboratory tap water (expressed as the number of micronucleated cells per thousand) carried out on pleurodele larvae, over a 8-month period between October 1985 and May 1986 (from JAYLET et al., 1987). +, positive result.

DATE	12.10.85 to 24.10 85		24 10 85 to 12 11 85		04 12 85 to 16.12.85		31.01.86 to 19 02.86		04.03.86 to 20 03 86		21.03.86 to 15 04.86		17.05 86 to 23 05 86	
	Filtered tap water	Tap water	Filtered tap water	Tap water	Filtered tap water	Tap water	Filtered tap water	Tap water	Filtered tap water	Tap water	Filtered tap water	Tap water	Filtered tap water	Tap water
Lower extreme	2	14	4	5	1	5	0	1	0	4	0	2	0	2
Lower quartile	6.5	20	5.5	14	5	10	2	5	2	10	1	3	4	7
Median	9	31	7	23	7	13	3	6.5	4	12.5	2	4	7	12.5
Upper quartile	13.5	45	9	41	11.5	17	4	8	5	19	3	4.5	8	16.5
Upper extreme	21	64	15	105	20	29	7	15	14	36	4	12	12	42
Mean	10.5	34 3	7.4	31 41	8.68	14 1	3 1	7 2	4 1	14 7	2 1	4 3	6.05	14.05
Results		+		+		+		+		+		+		+
Animal number	10	13	15	17	19	19	10	10	20	20	20	20	20	20
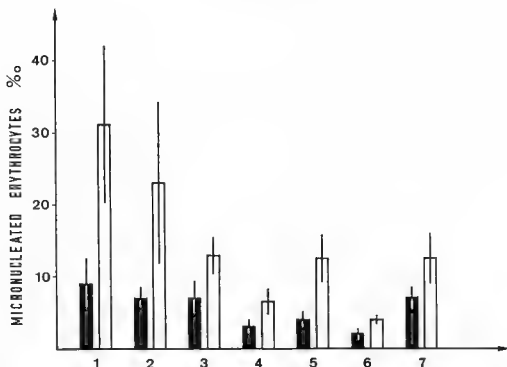
Fig. 3.   Histograms of median values obtained for various tests on laboratory drinking water samples. In black, tests using drinking water filtered over sand and carbon; in white, tests over the same period using non-filtered tap water. Error bars indicate 95 % confidence limits. Date of tests: N° 1, 12.10.85 to 24.10.85; N° 2, 24.10.85 to 12.11.85; N° 3, 04.12.85 to 16 12.85; N° 4, 21 01.86 to 19.02.86; N° 5, 04.03.86 to 20.03.86; N° 6, 21 03 86 to 15 04 86; N° 7, 17.05 86 to 23.05 86 (from JAYLET et al., 1987).

the non-chlorinated water. This procedure was repeated for 12 consecutive days. Control larvae were reared in non-chlorinated water throughout this period. In this case, results were also positive when the larvae were exposed for only 3 hours to the chlorine (0.2 ppm for 12 days) in the absence of food. The same experiment was carried out with monochloramine instead of sodium hypochlorite. The level of micronuclei increased with increasing concentration of monochloramine (0.05, 0.1 and 0.15 ppm) although only the 0.15 ppm concentration gave a statistically significant response. The results of these experiments indicated that free chlorine and monochloramine were responsible for the clastogen effect observed in newt larvae.

GAUTHIER et al. (1990) studied dechlorination effects of sodium hyposulfite ($Na_2S_2O_3$) on the genotoxic potential of chlorinated water with pleurodele larvae. The animals were reared both in reconstituted water and in reconstituted water containing 0.22, 0.56 and 1.12 ppm of chlorine (sodium hypochlorite). Chlorinated water samples were then fully dechlorinated by adding increasing concentrations of sodium hyposulfite (respectively 1, 2.5 and 5 ppm). For each test, the dechlorination was controlled by colorimetric dosage

of the residual chlorine. The same concentrations of sodium hyposulfite have been tested with the newt micronucleus test. In these experiments, no positive responses were observed, neither in dechlorination experiments nor in the water containing sodium hyposulfite alone. The authors concluded that sodium hyposulfite, a substance that is widely used in water treatment plants to control the concentration of chlorine in drinking water, was not genotoxic on pleurodele larvae. Moreover, dechlorination of water chlorinated with hypochlorite amounts, normally leading to clastogenic effects in the newt, led to the elimination of the genotoxicity.

These results confirmed previous results observed in water treatment plants by GAUTHIER et al. (1988) showing that pleurodele larvae reared in water samples taken at different treatment steps generally gave no positive responses after dechlorination with sodium hyposulfite, whereas positive responses were observed in chlorinated water samples partially dechlorinated by physical techniques.

Since ozone is also used to disinfect water (ozone has been increasingly used in Europe in view of its oxidizing, bleaching and deodorizing properties), JAYLET et al. (1990a) carried out other experiments in which pleurodele larvae were reared in river water containing different concentrations of ozone. After determination of the ozone demand in water samples of the river Seine, the following ozone concentrations were tested: 1/4 of the ozone demand in the raw water, 1/2, the overall and twice the ozone demand. The authors showed that treatment of Seine river water with low concentrations of ozone (corresponding to 1/4 of the ozone demand) led to a genotoxic effect, whereas ozonation at higher doses had no significant effect on the level of micronuclei induced. They concluded that ozonation of surface water with low doses of ozone may lead to genotoxic effects which are abolished by higher doses of ozone, thus confirming other results obtained with in vitro test systems (VAN HOOF, 1982; BOURBIGOT et al., 1986, KOOL & HRUBEC, 1986).

(2) According to our second hypothesis, genotoxic micropollutants may be substances produced by the action of chlorine on organic matter present in the raw water.

In order to examine this possibility, LECURIEUX et al. (1995a) studied the genotoxic effects of six halogenated acetonitriles identified in chlorinated waters (monochloro-, dichloro-, trichloro-, monobromo-, dibromo- and bromochloroacetonitrile), with three short-term assays: the SOS chromotest (QUILLARDET & HOFFNUNG, 1985; MARZIN et al., 1986; XU et al., 1989), the Ames fluctuation test (MARON & AMES , 1983; HUBBARD et al., 1984) and the amphibian micronucleus test on pleurodele larvae. In this study, clastogenic effects on peripheral blood erythrocytes of the animals were detected for all the six haloacetonitriles tested. The authors noted two structure-activity relationships. The genotoxic activity of haloacetonitriles containing bromine substituants appeared higher than the corresponding chlorinated acetonitriles and the clactogenic activity of the chlorinated acetonitriles increased with the number of chlorine substituents.

In the same way, LECURIEUX et al. (1995b) evaluated the genotoxicity of four trihalo-methanes (chloroform, bromodichloromethane, chlorodibromomethane and bromoform). The newt micronucleus assay detected a clastogenic effect on peripheral blood erythrocytes of pleurodele larvae for bromodichloromethane and bromoform. The authors noted that the presence of bromine substituent(s), generally led to significant genotoxic activity.

(3) Our last hypothesis on the origin of the genotoxic effects observed in drinking water suggested the responsibility of pollutants and/or micropollutants present in the raw water before treatment. It was important to find out whether the amphibian micronucleus test could be relevant to in vivo studies using natural waters taken directly from the aquatic medium.

MONITORING GENOTOXIC POLLUTION IN THE AQUATIC ENVIRONMENT

There are two different origins for water contamination. It may be due either to the presence of living organisms, viruses, bacteria and parasites, or it may have chemical origins. Within chemical contaminants, it is useful to distinguish solid particles in suspension, inorganic soluble and organic compounds, and radioactive substances. The genetic impact of pollutants corresponds to the overall effect of the various contaminants which interact within the aquatic environment and are all very unstable. Attempts to get a definition of the genotoxic load of polluted waters (waste waters, industrial effluents, etc.), were most often based on short-term bacterial assays, such as the Ames test, performed either directly in the waste water or after preconcentration of the organics. These assays performed directly in water often yield negative or ambiguous results (WEAVER et al., 1981). An additional problem is that loss of genotoxicity may occur when the sample is filtered for sterilization. Testing of concentrates will limit the scope of a survey to that part of the organic matter that can be recovered by concentration techniques. Many of the problems encountered with the in vitro assays may be circumvented with direct testing in aquatic organisms. In the past ten years, a number of tests were developed, either with plants or with aquatic animals (see the reviews of: CHOUROULINKOV & JAYLET, 1989; JAYLET & ZOLL, 1990; JAYLET et al., 1990b; ZOLL et al., 1990; GODET et al., 1993), which can potentially be used to assess the genotoxic potency of a waste water. The advantages are clear: there is no need to concentrate a sample or to sterilize it, and the tests can be carried out with intact animals, taking into account uptake and elimination, internal transport and metabolism.

Until now, few attempts have been made to incorporate in vivo genotoxicity assays in the quality assessment of effluents. A first comparison between different approaches, both from the technical point of view (sensitivity, practical use) and from the economic side, has been made by VAN DER GAAG et al. (1990). In order to make a comparison between several approaches for the assessment of genotoxic agents in wastewater, the authors characterized an industrial effluent with three different in vivo assays for genotoxicity, including sister chromatid exchange (SCE) induction in the fish *Nothobranchius rachowi* (VAN DER GAAG & VAN DE KERKHOFF, 1985; VAN DE KERKHOFF & VAN DER GAAG, 1985), and the formation of micronuclei in the amphibian *Pleurodeles waltl* and in the mussel *Mytilus edulis* (MAJONE et al., 1987; BRUNETTI et al., 1988; SCARPATO et al., 1990). The same effluent was tested in the Ames test and in the SOS chromotest. The genotoxicity assays were also performed in XAD-extracts of this effluent, as well as in the flow-through of the XAD columns. A number of routine chemical parameters have been determined in the waste water and in the effluents of the filter and of the XAD columns. Water samples were taken from an effluent of a biological waste water treatment unit receiving effluents from various petrochemical industries.

An 80-liter sample of waste water was taken at the discharge point of the biological waste water treatment plant. Twenty liters were used for direct in vivo testing and 15 liters were sterilized with gamma rays (25 kGy) for the tests on bacterial genotoxicity assays. To check the effect of sterilization, part of this sample was assayed with in vivo test systems. In order to assess the effectivity of methods that concentrate pollutants from the water, 45 liters of the sample were filtered over a 1 μm glass fiber filter, which was then passed over XAD 4, first at ambient pH and subsequently at pH 2 (according to the technique of NOORDSIJ et al., 1983). The organics were eluted from the filter and the XAD columns, and the final samples concentrated in ethanol. Part of the sample was collected after filtration and XAD pH 7 adsorption for testing in vivo and in vitro. After XAD pH 2 adsorption, the sample was neutralized with NaOH before testing.

Physico-chemical analysis before and after treatment showed that filtration of the industrial effluent affected neither the concentration of dissolved organic carbon nor the concentration of the various organohalides measured. Passing the industrial effluent through the XAD column at pH 7 only retained 25 % of the dissolved organic carbon; however it did retain the majority of the organohalides. Chromatography of the pH 7 XAD concentrate showed the presence of a large range of compounds which were lipophilic to moderately hydrophilic. The outflow from the first XAD column at pH 7 contained 75 % of the dissolved organic carbon and 50 % of the CaOX fraction (organohalides absorbed on carbon), but almost the entire EOX fraction (extractable organohalides) was retained by the resin. Adsorption on the second XAD column at pH 2 retained a further 23 % of the dissolved organic carbon of the sample and 30 % of the CaOX fraction. Analysis by HPLC showed a predominance of moderately hydrophylic compounds in this fraction. Finally, after the various treatments, the industrial effluent still contained more than 50 % of the dissolved organic carbon and about 30 % of the CaOX fraction.

Genotoxic effects were observed in waste water dilutions with the SCE assay with *Nothobranchius rachowi* and the micronucleus assays with *Pleurodeles waltl* and *Mytilus edulis*. The increase in SCE frequency and in incidence of micronucleated cells in pleurodele was still highly significant at the lowest concentration tested of 32 ml/l of dilution water. In the mussel, a significant increase only occurred at the highest concentration tested (100 ml/l). Filtration did not affect the in vivo genotoxicity A significant amount of the genotoxic effect observed in the SCE assay was present in the extract from a 1 μm glass fiber filter that was placed before the XAD columns. The genotoxicity in the SCE assay was not, however, altered by this filtration, suggesting that a part of the potential genotoxic effect of this effluent was not directly biologically available. XAD adsorption removed a major amount of the mutagenicity. Both in vivo tests (SCE in the fish and MN in amphibian), however, still detected a significant genotoxic effect in the flow-through of the XAD pH 2 column. The more hydrophilic part of the genotoxic activity could amount 15 to 40 % of the biologically available mutagenicity in this sample, according to the estimates from the SCE and micronucleus assays. Gamma-irradiation sterilization significantly reduced the genotoxic activity of the waste water in the SCE assay and in the amphibian micronucleus test.

The bacterial mutagenicity assays only detected effects in the different organic

concentrates Direct testing was carried out only in gamma-ray sterilized samples, but effects were not observed in the Ames test or in the SOS chromotest.

For the authors, this pilot study was a first clear proof that the analytical chemical techniques used to monitor waste waters only cover a part of the organic components that can be a potential risk to health: 30 to 50 % of the genotoxic effect present in the dissolved fraction of this specific waste water was not recovered on XAD. The yield is even lower if one accounts for the substantial amount of mutagenicity that was found in the filter extract, a non soluble fraction that was not directly biologically available. Although the genotoxic substances in this fraction are not directly taken up by aquatic animals, it is highly probable that these compounds will finally settle in sediments, and become available for bioconcentration in the long run if they are not degraded. Furthermore, in this study, the authors demonstrated that sterilization with gamma rays reduced the genotoxic effect of the waste water sample in both the SCE and micronucleus assay. These results introduce an uncertain aspect about those of the bacterial assays. the direct tests were carried out in gamma-ray treated waste water, and did not detect any mutagenicity at all. Considering the effectivity of in vivo test systems to detect genotoxic compounds in the industrial effluent studied, the authors concluded that the sensitivity of the in vivo assays was higher than that of the bacterial tests. The SCE assay and the micronucleus test could be carried out directly in dilutions of the sample, while a concentrate had to be made of the organics to assess the effect in the Ames test and the SOS chromotest. Furthermore, the costs and duration of the in vivo assays, if carried out on a routine basis, are not very different from those of bacterial assays.

Following this pilot study, GAUTHIER et al. (1993) published the results of a first environmental study using the micronucleus test on pleurodele larvae to detect the genotoxicity of waste waters and industrial effluents. In this study, pleurodele larvae were exposed to various types of industrial waste waters in order to estimate the ability of the micronucleus test system to reveal the genotoxic potency of polluted waters. The first test carried out on effluent from wool and leather industries (tannery effluent) showed that the newt micronucleus test was clearly able to demonstrate the genotoxicity of river water contaminated by this type of waste. Furthermore, negative results obtained with water samples taken at the same point and tested at the same concentration after that a large anti-pollution campaign has been in operation in the tanning and wool industries (waste reduction and purification) underlined the ability of the newt micronucleus test to play a potential role in assessing optimization of waste water treatment. A series of experiments carried out with an oil-refinery waste gave positive responses on the effluent sampled directly at the outlet, but not on effluent taken 300 m downstream. It could therefore be assumed that the genotoxic substances contained in the discharge have been diluted too much to bring about a positive result in the newt micronucleus test. It is also suggested that antagonistic relationships exist between certain substances contained in the waste water and substances present in the water receiving the waste (for example binding to humic matters or suspended solids). Furthermore, on the same effluent, induction of micronuclei was also observed when the animals were exposed to the samples for 8 days instead of 12. At the dilution of 250 ml/l, for both test durations, a positive response of similar amplitude was obtained, demonstrating that shorter test durations may be possible without substantial changes in the response. In the same study, other genotoxicity tests

carried out directly on a Dutch effluent taken in the river Rhine and on XAD extracts of the industrial effluent, also gave a direct demonstration of the genotoxic activity of waste from the petrochemical industry. The authors concluded that the experiments carried out with the newt micronucleus assay on industrial effluent of various origins underlined the ability of this method to detect the anthropogenic genotoxicity of polluted natural waters and to evaluate directly the impact of aquatic contamination on the exposed ecosystems. The simplicity of the test could allow its use in routine monitoring of, for example, the contamination of a river and in the framework of programs intended to reduce pollution. The newt micronucleus test was therefore considered as a welcome additional tool to help in decision making by the organizations responsible for supervising the discharge of waste into watercourses.

More recently, GODET (1994) compared the genotoxic potential of 8 effluents, using the Ames test and the newt micronucleus assay on pleurodele larvae. He obtained positive responses with 6 industrial effluents from various origins (metallurgy, chemistry) on pleurodele larvae and with the Ames test applied to metallic waste samples and to organic extracts of a paper mill effluent. Trying to identify genotoxic pollutants responsible of the positive responses observed with the newt micronucleus assay in industrial effluents containing Fe, Cr III, Cr VI and Zn, the author focused on the study of these metallic constituants on pleurodele larvae. Thus, Fe concentrations of 0.6 and 12.5 mg/l led to positive responses, whereas concentrations of 2.5 and 1 mg/l for Cr III and Cr VI respectively led only occasionally to positive responses. Previous genotoxic effects observed on pleurodele larvae with industrial effluents containing different mixtures of metallic pollutants were explained, considering the different types of effluents tested, either by the predominent effect of one of the metallic pollutants (Fe for example), or by the combined action of two of the metallic ions present in the effluent (Fe III and Cr VI). In conclusion, the author suggested the use of the sensitive newt micronucleus assay to reveal the possible synergistic effects of pollutants mixtures in water. He also proposed a new strategy to study the genotoxic potential of effluents, combining the Ames test and the amphibian micronucleus assay on pleurodele larvae, and suggested to modify the test procedure to optimize the detection of genotoxic effects in effluents and to help the development of this assay in a routine use.

In the current use of the amphibian micronucleus assay to evaluate genotoxic impact of waste water discharged in the environment, other types of effluents have been recently studied by GAUTHIER (1996). In the framework of an environmental study on the impact of roadway runfall effluents, the genotoxicity of 5 runfall samples has been evaluated during 6 different rainfall periods over a year, on pleurodele larvae. The author observed positive responses in animals exposed in 3 different effluents taken from the sampling site (a water retention tank receiving runfall effluents of a characterized roadway section). For each positive effluent, dose-effect responses have been obtained. Physicochemical analysis of the water samples have been carried out simultaneously to the genotoxicity assays, in order to try to correlate the genotoxic effects observed with the presence of some chemical substances. Even if it is not possible to conclude definitely from the data obtained, part of the genotoxic effects observed could probably be attributed to the high level of polycyclic aromatic hydrocarbons measured in the 3 positive effluents (from 84 to 188 ng/l compared to < 30 ng/l in the non-genotoxic water samples)

All the environmental studies reported previously have been carried out on pleurodele larvae. Until now, few studies of environmental interest have been devoted to *Xenopus laevis*, in spite of its rearing advantages and ease of use.

In 1987, LEHMAN & MILTENBURGER proposed the use of *Xenopus* larvae to study cytogenetic effects of toxicants in water. Micronucleus inducing effects of a waste water were analysed in poly- and monochromatic erythrocytes, with regard to exposure time. One of the strongest effects was observed in larvae exposed to the waste water during their entire larval development (3 months). Although these exposure conditions were incompatible with a routine use of a *Xenopus* micronucleus assay, these results were the first, from our knowledge, to report the use of platanna larvae for monitoring the genotoxic potential of a waste water.

In our laboratory, ZOLL-MOREUX (1995) developed a test procedure on *Xenopus* larvae inspired from the protocol of the newt micronucleus assay, that can be applied to environmental complex mixtures. This author compared the results obtained with the amphibian micronucleus test, using pleurodele and platanna larvae, on 5 chemicals of environmental interest and on 5 waste waters from various origins (urban waste and different industrial effluents). Four of the 5 tested chemicals were found genotoxic with the *Xenopus* micronucleus test, whereas only 3 were genotoxic in the newt micronucleus assay. Both organisms are considered quite equally sensitive (with a small advantage to *Xenopus* larvae) and the test procedures are similar. In conclusion, the author suggested that, even if it may be presumptuous to substitute the platanna for the pleurodele micronucleus assay in environmental genotoxicity studies (because of the lack of data), the undeniable advantages of the frog provide a good prospect for the use of this animal in future evaluation of genotoxic effects in the aquatic environment.

MECHANISMS INVOLVED IN THE FORMATION OF MICRONUCLEI IN AMPHIBIANS

Parallel to the in vivo detection of genotoxic effects observed with pure substances in water or with complex mixtures, several studies have been carried out, in our laboratory, to help the comprehension of the mechanisms of action involved in the formation of the micronucleated red blood cells observed in pleurodele larvae.

In 1987, FERNANDEZ & JAYLET demonstrated the antioxidant protection of the 2(3)-tert-butyl(4)hydroxyanisole (BHA), currently used as a food additive (E 320), against the clastogenic effects of BaP in pleurodele larvae. BHA was added to the water at concentrations of 0.5, 1 and 1.5 ppm. It reduced the clastogenic effects of BaP in the test larvae, with the most marked effect at a concentration of 0.5 ppm. In order to explain the results observed, the authors assumed that in the newt BHA influences various stages in the metabolic transformation and/or detoxication mechanisms of BaP. So, it was likely that metabolites of BaP and secondary reaction products (e.g. oxygenated free radicals) were responsible for the chromosome damage, leading to the appearance of micronuclei. BHA could thus act by attenuating the formation of these reactive intermediates or perhaps preventing them reaching their target(s) (DNA or mitotic apparatus).

Two years later, MARTY et al. (1989) investigated the effects of the indirect acting mutagen BaP, involving the action of hepatic cytochrome P 450 dependent monooxygenases, to explain the mechanisms controlling the formation of the active metabolites in newt larvae. The authors demonstrated that the pleurodele is capable of metabolizing BaP into hydroxylated products in a manner not so different from mammals or other aquatic organisms, of conjugating them with molecules implicated in the formation of polar derivatives to achieve their excretion and to respond to inducers of the 3-methylcholanthrene (3-MC) type. However, despite the metabolizing capacity in the pleurodele, accumulation of active metabolites of BaP can occur and is suggested as the main cause of the clastogenic effect of the compound.

MARTY et al (1992), working on the enzymatic activity of cytochrome P 450 forms induced in pleurodele after pretreatment by 3-MC or phenobarbital, showed that the newt is characterized by a lower level of hepatic cytochrome P 450-dependent activity than the rat. Variations of enzymatic activities according to sex and season were observed. Specific activities in newt were characterized by an almost complete insensitivity to induction by phenobarbital pretreatment. On the other hand, pretreatment by 3-MC resulted in an increase in the metabolism of several hydroxycoumarin and resorufin derivatives, similar to the effects observed in rat liver.

DISCUSSION

WHICH SPECIES IS THE BEST SUITED?

From the results presented above, it is possible to compare the performances of the different amphibian species used in the micronucleus assay to detect genotoxic substances in water. For the three species, the optimal treatment duration is quite similar and rearing the breeders does not present any particular problem. Urodele larvae eat exclusively live prey, whereas Xenopus tadpoles can be fed on dehydrated aquarium fish food. The interval between egg laying and testing is 6 weeks for urodeles and only 2 weeks for platanna, giving a technical advantage to the latter species However, recording micronucleated cells is somewhat easier in urodeles than in platanna, since red blood cells and micronuclei are larger in the former ones. Nevertheless, recording micronuclei for all three species is more straightforward than in the rodent micronucleus test. Pleurodele and Xenopus tests differ in sensitivity depending on the compound considered. For example, for cyclophosphamide the detection threshold is 0.5 ppm in pleurodele (FERNANDEZ et al., 1989) and 5 ppm in platanna. Similarly, for BaP the test is positive with 0.025 ppm in pleurodele (FERNANDEZ et al., 1989) and 0.06 ppm for platanna (VAN HUMMELEN et al., 1989). Conversely the detection threshold for methyl mercury is 2.5 ppb in platanna and 12 ppb in pleurodele (ZOLL et al., 1988).

All three species can demonstrate the genotoxicity of both direct and indirect mutagens (i.e. those requiring metabolic activation). Among the three species used for the test, it is not currently possible to state which is best suited for detection of genotoxic

substances in water. Nevertheless, recent work conducted by ZOLL-MOREUX (1995) with platanna on industrial effluents, together with the easy rearing conditions of the larvae, promise a future development of this amphibian to study the genotoxic activity of natural waters.

AMPHIBIANS: A SENSITIVE MODEL?

All our previous investigations on pure substances added to the rearing medium of the larvae have shown that the micronucleus test in amphibian is a sensitive and valuable model for the detection of genotoxic compounds in water. Various factors contribute to the high sensitivity of our biological model. The larvae strongly accumulate pollutants from the surrounding medium. This has been well demonstrated for compounds such as methyl mercury (ZOLL et al., 1988) and BaP (GRINFELD et al., 1986; MARTY et al., 1989). Studies carried out with various waste waters taken directly at industrial or urban sites confirm the ability of the amphibian micronucleus assay to reveal the presence of genotoxic substances discharged in the aquatic environment, even after dilution. In most cases studied, dose-related responses of the induction of micronucleated erythrocytes were observed. Furthermore, induction of micronuclei was also observed with test durations shorter than 12 days, without substantial changes in the response (GAUTHIER et al., 1993). The sensitivity of the method allows direct testing of water samples without the requirement for extraction or concentration of the micropollutants (techniques required for most in vitro studies) leading to the preparation of extracts which are not representative of the initial water samples (VAN DER GAAG et al., 1990). Because of the high sensitivity of our test system, genotoxic potential of drinking water has been detected. Water samples taken directly from the tap or taken at different treatment steps in water treatment plants were found genotoxic, whereas other test systems probably would not have revealed any mutagenic activity.

Presently, the amphibian micronucleus assay using pleurodele larvae is the only test system dedicated to an aquatic vertebrate and sufficiently sensitive to detect the genotoxic potential in drinking water samples.

IN VIVO AGAINST IN VITRO TEST-SYSTEMS?

We have shown (VAN DER GAAG et al., 1990) that the techniques of sterilization, extraction and concentration of organic micropollutants in water required for in vitro studies lead to the preparation of extracts which are not representative of the initial water sample. Moreover, the proportion of the genotoxicity arising from chemical pollutants transformed or neoformed during extraction, compared to that of the original sample, cannot be evaluated. These problems can be circumvented by the use of in vivo tests giving a direct evaluation of the genotoxicity of dilute effluents without the requirement for extraction or concentration of the micropollutants. Apart from this undeniable advantage, these in vivo tests present others which are also of considerable interest. Firstly, they use aquatic species which are exposed directly to the water to be tested. Also the species used are phylogenetically closer to man than are bacteria. Finally, the chromosomal damage is

evaluated in whole organisms and thus takes into account any interaction that might occur between micropollutants as well as phenomena of bioavailability and the overall metabolism of the animal. The responses obtained not only express the complex phenomena taking place in the whole organism, but also the interactions between the organism and its environment. Moreover, in vitro tests are not suited for detection of indirectly acting mutagens. To get around this problem, metabolic-activating systems are added to the bacterial culture. These are generally in the form of a microsomal preparation from rat liver, but can lead to problems in the interpretation of the results.

Nevertheless, the use of in vitro assays (tissue culture systems and bacterial tests) presents some other advantages. They are generally less time-consuming and expensive than the in vivo tests. They represent suitable early warning systems, although they cannot supplant the in vivo methods for more accurate evaluation of risks. Moreover, they score complementary endpoints of those of in vivo test systems. So, it is more and more proposed to associate in vitro and in vivo detection in a "test battery", able to reveal the various types of mutations (genic, chromosomal and genomic), to assess the majority of the genotoxic fraction in the water samples studied. For example, integration of the amphibian micronucleus test in a test battery for quality control of the water would help to the evaluation of risks to human health, as well as to the protection of aquatic ecosystems. The most commonly used tests, at present, are the in vitro test systems using bacteria, as they are both cheap and quick, and the in vivo test systems like the mouse micronucleus test, as it is well developed worldwide. However, this latter is not well adapted to aquatic environmental studies and was found less sensitive than the newt micronucleus assay for the detection of some carcinogens in water (LECURIEUX et al., 1992). In many cases, the risk from genotoxic agents in water is a hidden one. Although mutagenic micropollutants may not be lethal, they may alter the natural equilibrium in subtle ways or even sterilize fragile species. An enhancement in the rate of mutation may also accelerate the process of evolution, leading to a domination of certain species to the detriment of others, although here the precise risk is hard to estimate. In any event, extrapolation of the tests' results to the real-word situation represents an exercise in judgment.

FUTURE DEVELOPMENT AREAS

Presently, one of the research axes developed in our laboratory deals with fundamental preoccupations. Recent research (unpublished results) has shown the possibility to detect aneugenic effects of some genotoxic substances using the amphibian micronucleus assay. Knowing the close relations between aneuploidy and the initiation of cancers, it seems important to be able to detect the presence of these specific genotoxic substances in the aquatic environment. It is clear that the future development of the amphibian micronucleus test will find its way in environmental research. Up to now, the pilot studies carried out with this test system on waste waters of various origins underline the ability of this method to detect the anthropogenic genotoxicity of polluted natural waters and to evaluate directly the impact of aquatic contamination on the ecosystems exposed. The simplicity of the test could allow its use in routine monitoring of, for example, the contamination of a river and in the evaluation of programs of installations

intended to reduce pollution. However, in its current form, this in vivo test system remains a relatively heavy and costly technique, limiting its general diffusion in spite of its ease of use. For this reason, it seems now necessary to improve the feasibility of the test by shortening the exposure time of the larvae and helping the scoring of the micronucleated cells in analyzing the blood smears with a computer. We are therefore trying to develop in our laboratory an automated method of scoring the micronucleated red blood cells with a computerized imaging system. Another line of research consists in the extension of the application field of the amphibian micronucleus assay. Up to now, only the aquatic compartment has been evaluated using this method. However, it is well known that genotoxic pollutants, even if they transit in the aquatic compartment, often find their way in soils or sediments. For instance, the results obtained by VAN DER GAAG et al. (1990) indicate that part of the genotoxicity in natural water samples was absorbed onto particulate matter and would only become available to biota during intensive contact, such as may occur in sediment. In this respect, the genotoxicity may have a serious toxicological relevance in sedimentation areas. To explore the genotoxicity of contaminated sediments or soils, using our laboratory test system, new approaches have to be perfected (new exposure conditions, lixiviation of soil samples).

AMPHIBIANS: WELCOME GENOTOXICITY BIOINDICATORS?

Finally, all the experiments carried out over the last decade on the induction of micronuclei in the red blood cells of pleurodele, axolotl and platanna larvae confirm our initial choice of these animals as efficient laboratory detectors of the hidden risks in the aquatic environment. Up to now, few studies have reported the use of amphibians as real in situ bioindicators of the genotoxic potential of natural waters. Some trials have been undertaken on other aquatic vertebrates, like fishes, representing a potential material for the setting of the micronucleus test, but at the present time the fish micronucleus assay does not seem capable of constituting a routine test to study the genotoxicity of water (HOOFTMAN & VINK, 1981, HOOFTMAN & DE RAAT, 1982; CARRASCO et al., 1990). Programs designed to protect the environment require methods for evaluating the genotoxic hazards to which the various populations constituting aquatic ecosystems are exposed. Since such hazards are water-borne, laboratory tests need to be developed that assess directly the genotoxicity of water, effluents and substances or preparations to aquatic organisms. However, the in vivo tests on aquatic organisms are not yet widely accepted, they require more backing to play a major role. They can already be useful at this time, for instance by including them in Toxicity Reduction Evaluation (TRE) such as they are advised or prescribed already by different governmental agencies (ANONYMOUS, 1989, 1990; VAN DER GAAG, 1991).

DECLINING AMPHIBIAN POPULATIONS: A WAY OF EXPLANATION?

The populations of many amphibian species, in various habitats worldwide, appear to be in severe decline (WAKE et al., 1991). There is no known single cause for the declines, but their widespread distribution suggests involvement of both global and local agents (BLAUSTEIN et al., 1994b; DUBOIS, 1994), among which habitat destruction, conversion of

agricultural lands from traditional uses, introduction of predators and competitors, pollution from pesticides (BERRILL et al., 1993, 1994), mining and logging, acid precipitation (POUGH & WILSON, 1977; TOME & POUGH, 1982; COOK, 1983), increased levels of ultraviolet irradiation (BLAUSTEIN et al., 1994a), consumption by humans and global climate change.

In any way, the increasing presence of genotoxic agents in many natural aquatic media could be considered as a factor contributing to the decline of amphibian populations. Indeed, the mutagenic risk may affect any cell in the organism. Mutation in a somatic cell may trigger a process leading to carcinogenesis. Mutagenic agents also exert their action on germ cells. If the toxicity is severe and many cells are affected, there may be a lowered or even temporary loss in fertility. If a gamete with a genetic anomaly contributes to the formation of a zygote, the disorder created in the hereditary material may be serious enough to lead to the death of the embryo. Although a genetic anomaly that is compatible with survival of the organism frequently leads to an immediately obvious anomaly in the phenotype, the effect may be differed and only become apparent in future generations. This is often the case with equilibrated chromosomal rearrangements and recessive mutations.

Until now, few studies have been carried out to monitor the behavior of our three test-organisms after exposure. GRIENFELD et al. (1986) observed an increase in frequency of micronuclei in pleurodeles larvae 6 days after the beginning of exposure (12 to 48 hours) to BaP (0.5 ppm) and a progressive decrease of the micronucleated cells from day 6 to day 12. The decrease could be interpreted as the result of several phenomena: the progressive dilution of the micronucleated erythrocytes with normal cells, the destruction of the micronucleated cells or of the micronuclei in the damaged cells. What about the real impact of exposure to genotoxic substances in amphibian erythrocytes with other exposure times, in other cellular types, especially those involved in the reproduction, in the whole organism and on the population itself?

At this time, there is no clear evidence to answer these questions on our test-organisms, because of the lack of data. Most of the work remains to be done and will constitute the challenge for batrachologists and toxicologists over the next decades, as expressed by WAKE (1991): "Amphibians may serve usefully as bioindicators, organisms that convey information on the state of health of environments. How to read the message and what to do about it, are timely challenges to scientists and to the public."

RÉSUMÉ

Cet article constitue une revue des travaux réalisés depuis une dizaine d'années sur la détection du pouvoir génotoxique des milieux aquatiques (eaux douces) à l'aide de larves d'amphibiens. Trois espèces ont été plus particulièrement utilisées pour réaliser ces études. Il s'agit de deux urodèles, le pleurodèle (*Pleurodeles waltl*) et l'axolotl (*Ambystoma mexicanum*), ainsi que d'un anoure, le xénope d'Afrique du Sud (*Xenopus laevis*). La technique mise au point sur les larves de ces trois espèces d'amphibiens au Centre de Biologie du Développement de Toulouse, France, permet de révéler directement la

présence de substances génotoxiques dans l'eau d'élevage des animaux en mesurant le niveau d'induction de micronoyaux dans les érythrocytes des larves exposées à des substances pures, à des agents physiques ou à des mélanges complexes de substances, tels que des eaux de boisson, des eaux de surface, des rejets industriels et domestiques, ou tout effluent aqueux.

Après une présentation du protocole des essais et des conditions d'élevage des animaux, les résultats obtenus avec le test des micronoyaux sur les larves d'amphibiens sont rapportés en respectant le déroulement chronologique des différents événements qui ont abouti, d'une part à la publication de l'essai sous sa forme normalisée en 1992, et d'autre part aux orientations actuelles des recherches menées dans le domaine de l'écotoxicologie génétique in vivo des milieux aquatiques, où les amphibiens, grâce à l'ensemble des travaux rapportés dans le présent article, tiennent une place prépondérante.

Le choix des orientations de travail futures est ensuite discuté en termes de validité de l'utilisation des amphibiens comme bioindicateurs de génotoxicité en milieu aquatique, considérant les nombreux avantages de ce modèle et sa complémentarité avec les tests in vitro qui sont les plus utilisés dans ce domaine

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Morfología de los oocitos en diplotene de *Melanophryniscus stelzneri* (Weyenbergh, 1875) (Anura, Bufonidae)

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Five stages of the oogenesis, with eight substages of the diplotene oocyte development, are identified applying histological and transmission electron microscopy techniques. Based on the internal morphology of the oocytes, the following features were taken into account: yolk vesicles, yolk platelets, cortical granules and pigment appearance, morphological variations of the nuclear envelope and nucleoli.

INTRODUCCIÓN

La distribución geográfica de las especies del género *Melanophryniscus* Gallardo, 1961 se halla restringida a Sudamérica. CEI (1980) mencionó la existencia de cuatro subespecies de *M. stelzneri*, de las cuales *M. s. stelzneri* habita en las sierras de Córdoba y San Luis

Es frecuente hallar ejemplares de *M. s. stelzneri* activos durante el día, luego de fuertes lluvias, cerca de arroyos o pequeños cuerpos de agua de zonas serranas, entre los 900 y 2.000 m. La época de reproducción de la especie abarca desde mediados de octubre a fines del verano (GALLARDO, 1987).

Debido a lo restringido de su distribución y a que estos animales son comercializados como mascotas, se hallan entre las especies que la IUCN (Unión Internacional para la Conservación de la Naturaleza) y la FVSA (Fundación Vida Silvestre Argentina) coinciden en llamar *raras* (especies sujetas a riesgo) y *comercialmente amenazadas*, respectivamente. La extracción de estos animales de su medio ambiente, llevada a cabo en forma indiscriminada, podría afectar, por ejemplo, la abundancia y distribución de las poblaciones de *M. s. stelzneri* en los ambientes serranos.

El estudio de la oogénesis en los anuros ha sido objeto de numerosas investigaciones, que en su mayoría han destacado la variación morfológica de los oocitos en el ovario adulto durante el período reproductivo de la especie. La caracterización histológica de los oocitos del ovario se ha efectuado en diversas especies. Se pueden mencionar, por ejemplo, los trabajos de BOUIN (1901) en *Rana sylvatica*, KING (1908) en *Bufo lentiginosus*, ALLENDE (1938), VALDEZ TOLEDO & PISANÓ (1980) y ECHEVERRÍA (1988) en *Bufo arenarum*, KEMP

(1953) en *Rana pipiens*, IWASAWA (1969) en *Rana ornativentris*, DUMONT (1972) en *Xenopus laevis*, LAMOTTE et al. (1973) en *Nectophrynoides occidentalis*, y IWASAWA et al. (1987) en *Rana nigromaculata*.

El proceso más evidente de la maduración de los oocitos es la vitelogénesis. Se han propuesto distintos modelos para explicar la formación del vitelo. Tanto WALLACE (1985) como DUMONT (1972) han expresado que el principal proceso de adquisición de vitelo en los oocitos de *Xenopus laevis* es la micropinocitosis. OPRESKO et al. (1980) propusieron un modelo de compartimentalización endocítica de las proteínas en oocitos de *X. laevis*. En varias especies de anfibios se ha descrito la formación de inclusiones cristalinas dentro de las mitocondrias en oocitos que fueron interpretadas como progenitoras de los cristales formados dentro de las plaquetas (WARD, 1962; KARASAKI, 1963; WALLACE & KARASAKI, 1963; LANZAVECCHIA, 1968; WALLACE & DUMONT, 1968; MASSOVER, 1971; WALLACE et al., 1972). Años después, WARD et al. (1985) concluyeron que las formaciones cristalinas no eran precursoras del vitelo. En oocitos jóvenes se ha observado la presencia de cuerpos multivesiculares (LANZAVECCHIA, 1968) que han sido relacionados con la síntesis endógena de vitelo (WALLACE, 1985). VILLECCO et al. (1992) han observado en *Ceratophrys cranwelli* la formación de cuerpos multivesiculares como precursores de las plaquetas vitelinas, que se originan de la fusión de endosomas.

Es una característica sobresaliente de los oocitos de anfibios la presencia de una gran cantidad de nucléolos en ciertas etapas del crecimiento. Estos nucléolos son formados a partir de genes del ADN ribosomal, que se hallan amplificados en los oocitos de anfibios (BROWN & DAWID, 1968; AMALDI et al., 1973; SCHEER & DABAWALLE, 1985).

En *Rana temporaria* y *Bufo arenarum*, al cabo de tres años los oocitos finalizan su desarrollo y la hembra alcanza la madurez sexual (GRANT, 1953; ECHEVERRÍA, 1988). VALDEZ TOLEDO & PISANÓ (1980) han convenido en clasificar las fases de la oogénesis de *Bufo arenarum* en cinco estadios. Se pueden distinguir cuatro estadios inmaduros (previtelogénesis, vitelogénesis temprana, vitelogénesis tardía y oocito en auxocitosis) que conducen al estado final postvitelogénico de oocito ovulable (ECHEVERRÍA, 1988).

El propósito de este trabajo radica en caracterizar los estadios de la oogénesis en *M. s. stelzneri* como primeros pasos hacia el conocimiento de la biología reproductiva de la especie en cuestión que, sumado a otras investigaciones, permitirían implementar una buena y eficiente estrategia de conservación de *M. s. stelzneri*.

MATERIAL Y MÉTODOS

Se capturaron 17 hembras de *Melanophryniscus stelzneri* en las localidades de Tanti, provincia de Córdoba y El Trapiche, provincia de San Luis, en los meses de octubre, diciembre y febrero.

Los animales fueron adormecidos con éter y luego colocados con el abdomen abierto en distintos fijadores.

La muestra constó de trozos de ovario de aproximadamente 5 mm × 5 mm de espesor y de oocitos aislados de cada una de las hembras.

A fin de conocer el aspecto general de los oocitos se realizó el examen con microscopio estereoscópico. Se implementaron dos técnicas complementarias

Técnicas histológicas generales (según Echeverría, 1988)

    − Líquidos fijadores. formol al 10 %; solución de Bouin; líquido de Zenker.

    − Inclusión: en parafina 56-58°.

    − Técnicas de coloración e histoquímicas (Drury & Wallington, 1967; Pearse, 1972): hematoxilina - eosina, tricrómico de Masson, coloración de Mann; reacción del ácido peryódico Schiff (PAS) - hematoxilina; PAS - azul Alcián (pH 3,5); PAS - diastasa; verde de metilo-pironina.

Técnicas de microscopía electrónica de transmisión

Fijación con glutaraldehído 3 % en buffer cacodilato de sodio 0,1 M (pH 7,2-7,4), refijación con tetróxido de osmio 2 %; coloración en bloque con acetato de uranilo 2 %, deshidratación en alcoholes de graduación ascendente, con líquido intermediario óxido de propileno; impregnación e inclusión en Polybed.

Se realizaron cortes de 500 Å que se recogieron en grillas y se colorearon sucesivamente con solución de Reynolds (acetato de uranilo al 2 %, citrato de plomo) según Reynolds (1963, fide Mercer & Birbeck, 1979), y nitrato de bismuto en solución de tartrato de sodio.

Se han establecido los estadios de desarrollo de los oocitos según Echeverría (1988), teniendo en cuenta diferentes características anatómicas e histológicas que se mencionan en la Tabla I.

Los estadios de la oogénesis se identificaron a partir de las observaciones de los oocitos desprovistos de la teca externa y de la serosa, y se dividieron en subestadios para facilitar las observaciones del progreso de la oogénesis.

RESULTADOS

Estadio 1. Previtelogénesis

Son los oocitos de tamaño más pequeño (subtipo a), entre 22 y 50 µm de diámetro. Se ubican por fuera de la teca externa, cubiertos por la serosa que envuelve al ovario. Presentan un citoplasma transparente a través del cual se observa el núcleo ocupando gran parte de la célula. El contenido citoplasmático, de apariencia granular, es levemente basófilo. El núcleo es esférico y central, con la membrana lisa. En la periferia del núcleo se disponen prominentes nucléolos esferoidales y de contorno irregular, en un número que

Tabla I. Caracteres morfológicos y métricos para la identificación de los estadios del desarrollo de los oocitos de *Melanophryniscus stezneri* en cortes histológicos. A, anulares; B, bipartitos; C, central; E, esferoidales; G, plegada; L, lisa; O, ondulada; S, saculada; P, polarizado, V, vacuolados; +, presencia; #, escasos y aislados; *, ordenados; -, ausencia.

Estadio	Subtipo	Diámetro mínimo-máximo	Núcleo			Citoplasma			Membrana vitelina
			Posición	Membrana nuclear	Nucléolos	Vitelo	Pigmento	Gránulos corticales	
1	a	22-50	C	L	E	-	-	-	-
	b	50-150	C	L	E	-	-	-	-
	c	150-270	C	O	E	-	-	-	±
2	a	270-310	C	S	B	-	-	-	+
	b	310-350	C	S	B	+	-	-	+
3	a	350-650	C	S	A, B, E	+	+	+ #	+
	b	650-740	C	S	B y E	+	+	+ #	+
	c	750-900	P leve	S	B y V	+	P +	+ #	+
4		900-1200	P	G	E y V	P +	P +	+ *	+
5		1200-1400	P	G	E y V	+	+	+ *	+

varía entre cinco y diez. El nucleoplasma presenta densas granulaciones intensamente coloreadas que permiten asociarlas con el estado de diplotene temprana de la profase de la meiosis (fig. 1). Rodeando a cada oocito, en contacto con la membrana celular se disponen las células foliculares de núcleo prominente.

A partir de los 50 μm de diámetro los oocitos se hallan rodeados por las tecas. En los oocitos cuyos diámetros están comprendidos entre los 50 y 150 μm (subtipo *b*), la basofilia del citoplasma se ve incrementada notablemente. El núcleo presenta en la zona periférica una mayor cantidad de nucléolos. A medida que el diámetro celular aumenta, los nucléolos tienden a disminuir el volumen. La membrana nuclear manifiesta leves ondulaciones. Ocupando todo el volumen del núcleo se observan los cromosomas en diplotene temprano (fig. 2). Aumentan las células foliculares alrededor de los oocitos

Los oocitos que presentan un diámetro comprendido entre los 150 y 270 μm (subtipo *c*), se observan levemente opacos. En los cortes histológicos el citoplasma evidencia zonas densas, continuas o irregulares alrededor de núcleo, con gran afinidad por los colorantes básicos, que denominamos zonas basófilas del citoplasma. El núcleo conserva la posición central. La membrana nuclear presenta ondulaciones que se hacen más notorias a medida que aumenta el tamaño del oocito. En la zona cortical del núcleo es evidente el aumento del número de los nucléolos esferoidales. Los cromosomas que pierden su afinidad por los colorantes básicos pueden observarse en el área central del núcleo. La membrana vitelina comienza a visualizarse como una delgada y discontinua envoltura PAS positiva rodeando a los oocitos, por debajo de las células foliculares.

ESTADIO 2. VITELOGÉNESIS TEMPRANA

Los oocitos que inician el proceso de vitelogénesis y cuyos diámetros están comprendidos entre 270 y 310 μm (subtipo *a*) se caracterizan por un color blanco opaco El citoplasma exhibe vesículas en la zona periférica, que dan reacción PAS positiva, y contactan con la membrana celular (figs. 3-4). Zonas basófilas se disgregan alrededor del núcleo. La vesícula germinal manifiesta una membrana excesivamente saculada que se proyecta hacia el citoplasma. Los nucléolos se disponen ocupando la mayor parte del núcleo. Son evidentes nucléolos bipartitos, constituidos por dos zonas de distinta afinidad por el colorante. Las zonas menos densas pueden repetirse en la estructura del mismo nucléolo y disponerse alternadamente en forma lineal o como brotes sobre la esfera más densa, generalmente de mayor volúmen (fig. 5). La membrana vitelina continúa su desarrollo envolviendo totalmente al oocito.

Los oocitos de color ocre claro con un diámetro que oscila entre los 310 y 350 μm conforman el subtipo *b*. Las observaciones histológicas ponen de manifiesto la presencia de vitelo de apariencia granular en la zona cortical del oocito, que se destaca del resto del citoplasma por su gran afinidad por los colorantes ácidos (fig. 6). Se ha estudiado con MET esta zona del oocito, pudiéndose identificar pequeñas plaquetas vitelinas y vesículas cuyo contenido central es de forma irregular (*premelanosomas*, según DUMONT, 1972), dispersas en el citoplasma. La membrana oocitaria manifiesta un gran desarrollo de microvellosidades que se proyectan hacia la membrana vitelina.
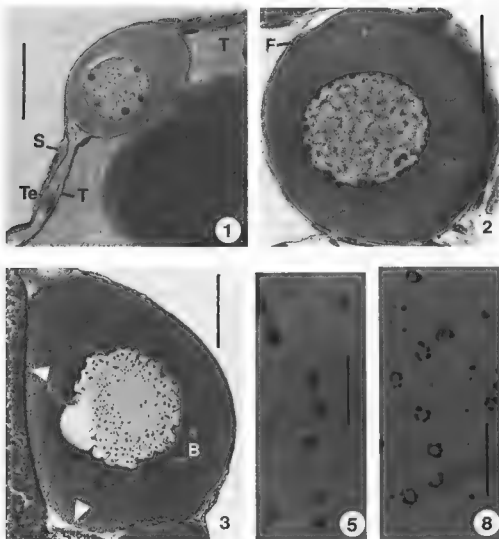
Fig 1. – Oocito de estadio 1a. S, serosa; T, teca interna, Te, teca externa. Tricrómico de Masson. Escala: 20 µm.

Fig 2. Vista general de oocito de estadio 1b. F, núcleo de célula folicular. Tricrómico de Masson. Escala: 30 µm.

Fig. 3. Vista general de oocito de estadio 2a. Triángulos blancos, citoplasma periférico. B, zonas basófilas. Tricrómico de Masson. Escala. 100 µm.

Fig. 5. Nucléolos bipartitos en el nucleo de un oocito de estadio 2a. Verde de metilo-pironina. Escala: 20 µm.

Fig. 8. – Nucléolos en anillo. Azul de metileno. Escala 20 µm.
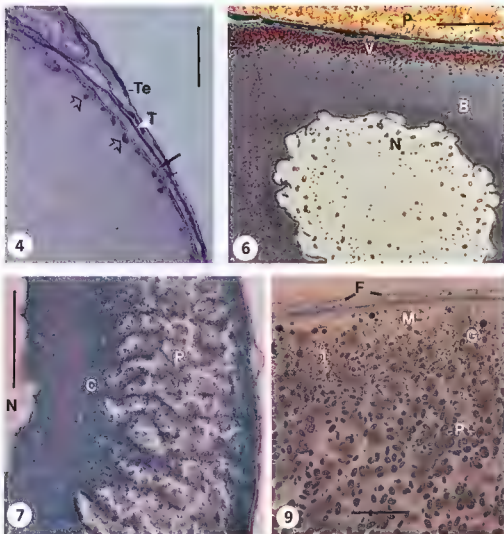
Fig. 4. – Citoplasma periférico de oocito de estadio 2a con zonas irregulares PAS positivas (sagitas negras cortas) adyacentes a la membrana vitelina (sagita negra larga) T, teca interna, Te, teca externa. PAS - azul Alcián. Escala: 20µm.

Fig. 6 — Sección de un oocito de estadio 2b con una banda periférica de vitelo (V). B, zonas basófilas, N, núcleo, P, plaquetas vitelinas de un oocito adyacente en vitelogénesis tardía Tricrómico de Masson. Escala: 50 µm.

Fig. 7 — Oocito de estadio 3a. Plaquetas vitelinas (P) invadiendo el citoplasma (C) N, núcleo Coloración de Mann. Escala: 50 µm

Fig. 9.   Corte semifino de un oocito de estadio 3a coloreado con azul de metileno. F, núcleo de células foliculares; G, gránulo cortical, I, pigmento, M, membrana vitelina, P, plaquetas vitelinas. Escala: 20 µm.

ESTADIO 3. VITELOGÉNESIS ACTIVA

A partir de los 350 y hasta 650 µm, el vitelo se encuentra organizado en plaquetas vitelinas, ocupando las dos terceras partes del citoplasma (subtipo *a*). A medida que progresa la vitelogénesis se observa un gradiente en la forma y tamaño de las plaquetas; las más pequeñas se ubican en la zona subcortical de la célula, mientras que las más internas son de mayor tamaño. El citoplasma sin plaquetas queda restringido a una franja perinuclear (fig. 7). Las vesículas PAS positivas, localizadas en la zona más externa de la banda de vitelo, aumentan en número y tamaño. El núcleo conserva las características del estadio anterior y los nucléolos se hallan distribuidos en todo el volumen nuclear; aparecen nucléolos con distinta morfología. Se identifican tres tipos: nucléolos bipartitos; nucléolos en forma de anillo, constituidos por cuentas, que se observan con frecuencia en la zona más central (fig. 8); y nucléolos esferoidales simples periféricos. En la zona cortical del citoplasma se evidencia la presencia de gránulos de pigmento y de gránulos corticales de 2,5 µm, cercanos a la membrana celular (figs. 9-10); éstos son esféricos y su contenido es homogéneo.

Los oocitos cuyo diámetro oscila entre los 650 y 740 µm se caracterizan por presentar un color castaño en toda su superficie (subtipo *b*). El citoplasma se halla invadido por plaquetas vitelinas, las cuales alcanzan el núcleo. La franja perinuclear de citoplasma basófilo queda reducida a un anillo de grosor variable inmerso en el vitelo. Los gránulos de pigmento se distribuyen homogéneamente en toda la región cortical de la célula (fig. 11). La disposición de los nucléolos se modifica. Los nucléolos bipartitos se agrupan en el centro del núcleo y nucléolos esferoidales se localizan en la periferia.

A partir de los 750 µm de diámetro, comienza a distinguirse un desplazamiento gradual del pigmento (subtipo *c*). Las plaquetas vitelinas de mayor tamaño se ubican en el centro del hemisferio vegetativo, por debajo de la vesícula germinal. Los gránulos corticales se hallan distribuidos en una amplia franja de citoplasma cortical entre las plaquetas vitelinas. El núcleo migra hacia la zona más pigmentada del oocito, el hemisferio animal. La morfología del mismo se modifica de esférica a ovoal, y su eje mayor se dispone paralelo al ecuador del oocito. En la zona periférica del núcleo aparecen nucléolos esféricos de aspecto vacuolar o *vacuolados* (según DUMONT, 1972) que contienen áreas de material de menor afinidad por los colorantes básicos.

ESTADIO 4. VITELOGÉNESIS TARDÍA

Estos oocitos presentan una visible polarización del pigmento. El hemisferio animal, castaño oscuro, contrasta con el hemisferio vegetativo de color castaño claro. El diámetro está comprendido entre los 900 y 1200 µm. La figura 12 muestra una vista general de un corte de un oocito de estadio 4. El citoplasma está totalmente ocupado por plaquetas vitelinas que se distribuyen según un gradiente de tamaño. Alrededor del núcleo son evidentes las de menor tamaño. La membrana nuclear manifiesta una serie de pliegues hacia el interior del núcleo que se hacen más numerosos y profundos hacia el lado del hemisferio vegetativo. Los nucléolos periféricos se observan con mayor frecuencia entre los pliegues de la membrana nuclear. En el centro se agrupan nucléolos esferoidales de menor
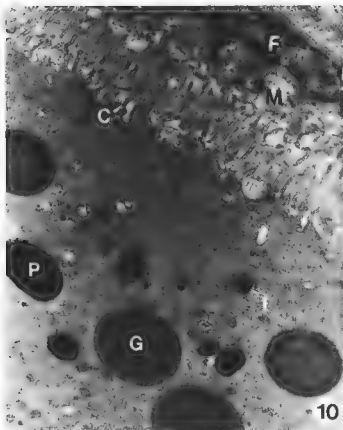
Fig. 10 — Región cortical de un oocito en estadio 3a. C, membrana citoplasmática; F, núcleo de célula folicular; G, gránulo cortical, I, pigmento, M, membrana vitelina; P, plaqueta vitelina. Aumento: 7000 ×.

tamaño y de apariencia homogénea. Los cromosomas comienzan a visualizarse en la zona central al aumentar su afinidad por los colorantes básicos. El núcleo presenta un halo subnuclear de material basófilo y PAS positivo, en íntimo contacto con los pliegues de la membrana. El tratamiento con diastasa produce una reacción PAS negativa. Con MET se observa que está constituído por mitocondrias y un material electro-opaco que podría asociarse con glucógeno y ribosomas (fig. 13). Los gránulos corticales se disponen ordenados debajo de la membrana plasmática. Presentan un diámetro aproximado de 3 μm. En el hemisferio animal, los gránulos de pigmento se hallan por debajo de los gránulos corticales.

ESTADIO 5. OOCITO OVULABLE O MADURO

En estos oocitos, cuyos diámetros oscilan entre 1200 y 1400 μm, se acentúa la diferencia de coloración entre el hemisferio animal y el vegetativo. El hemisferio animal,
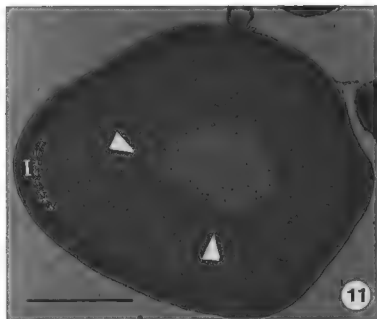
Fig. 11. -- Vista general de oocito de estadio 3b. Pigmento (I) distribuído en la región cortical y banda de material perinuclear basófilo (triángulos blancos). Hematoxilina de Harris - eosina Escala: 200 μm.
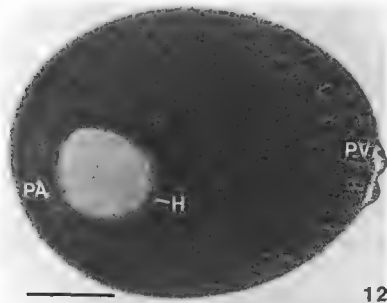
Fig. 12. - Vista general de un oocito de estadio 4 Polo animal (PA) con pigmento H, halo subnuclear; PV, polo vegetativo Hematoxilina de Harris - eosina. Escala: 300 μm.
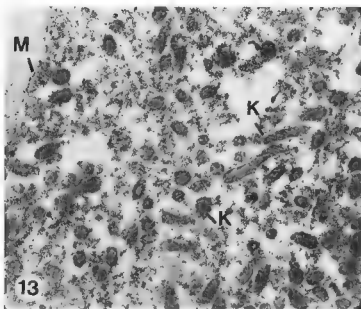
Fig. 13. – Citoplasma subnuclear de oocito de estadio 4. K, mitocondrias; M, membrana nuclear. Aumento: 22500 ×.
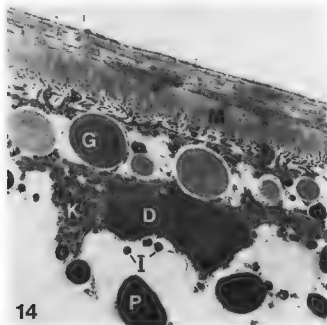
Fig. 14. – Región cortical del polo animal de un oocito de estadio 5. D, complejo de Golgi; G, gránulo cortical; I, gránulos de pigmento; K, mitocondrias, M, membrana vitelina, P, plaqueta vitelina. Aumento: 6000 ×.

de color castaño oscuro, presenta una zona circular en el polo animal con menos pigmento. La disposición de las plaquetas vitelinas en el citoplasma es semejante a la del estadio 4. En el hemisferio vegetativo, las plaquetas alcanzan una longitud máxima de 10 μm. El anillo subnuclear persiste en estos oocitos. En el núcleo se observa una reducción del número de nucléolos. Los nucléolos periféricos aumentan de tamaño alcanzando un diámetro de 9 μm. En este estadio los cromosomas son poco visibles con las técnicas de coloración utilizadas. Los gránulos corticales conservan la forma esférica, se hallan situados por debajo de la membrana celular y alcanzan un diámetro de 4 μm. Las microvellosidades oocitarias se hallan retraídas y el espacio perivitelino se ve aumentado (fig. 14).

DISCUSIÓN

Para establecer los estadios de la oogénesis se han utilizado distintos criterios. En *Rana pipiens*, están basados principalmente en el tamaño del oocito o en la cantidad y distribución del vitelo y del pigmento (GRANT, 1953; KEMP, 1953), aunque otros autores utilizaron solamente la morfología de los cromosomas (DURYEE, 1950). En *Xenopus laevis*, la identificación de los estadios se basa en el aspecto externo de los oocitos y en las observaciones histológicas de los mismos (DUMONT, 1972; CALLEN, 1984). En este trabajo se han considerado los caracteres morfológicos externos e internos de los oocitos, integrando las diferentes propuestas aludidas, que permite establecer cinco estadios de la oogénesis con ocho subestadios que se sintetizan en la Tabla I. Los cinco estadios se consideran suficientes y convenientes para establecer el curso de la oogénesis.

El pigmento ha sido considerado como un producto catabólico y comienza a aparecer cuanto más activo es el metabolismo del oocito (VALDEZ TOLEDO & PISANÓ, 1980). En *Bufo arenarum*, la formación del pigmento se evidencia en el estadio de vitelogénesis primaria, distribuido en todo el citoplasma (VALDEZ TOLEDO & PISANÓ, 1980; ECHEVERRÍA, 1988). La acumulación de la melanina en oocitos de *Xenopus laevis* se incrementa durante los estadios de vitelogénesis temprana y tardía hasta el estadio V (DUMONT, 1972). En *Melanophryniscus stelzneri*, el pigmento es escaso y de color castaño a diferencia de las especies anteriormente mencionadas, en las cuales éste es negro y abundante. El pigmento aparece al comienzo del estadio de vitelogénesis activa distribuido homogéneamente en el citoplasma cortical. A partir del estadio 4 se acumula en el hemisferio animal, siendo característica la presencia de la mancha polar en el polo animal en el estadio 5

El inicio del proceso de vitelogénesis se evidencia en *Melanophryniscus stelzneri* en el estadio de vitelogénesis temprana (2.a) con la aparición de pequeñas vesículas en la zona cortical del citoplasma. Las plaquetas vitelinas se organizan al finalizar la vitelogénesis temprana (estadio 2.b), ubicándose en la periferia de la célula. Todas las plaquetas vitelinas son homogéneas independientemente de su ubicación y tamaño, es decir, no se distingue en ellas un cuerpo principal cristalino como se describe en las especies estudiadas (WISCHNITZER, 1957). DUMONT (1972) y OPRESKO et al. (1980) proponen que el aumento de tamaño de las plaquetas vitelinas es ocasionado por la fusión de vesículas endocíticas a las plaquetas ya formadas. En *M. stelzneri*, se ha podido observar con microscopio

electrónico de transmisión grupos de pequeñas plaquetas que se contactan; este hecho podría interpretarse como un proceso de fusión. El crecimiento de las plaquetas posiblemente se produzca a expensas del material presente en el citoplasma.

La mayoría de los autores asocia la aparición de las vesículas de vitelo y de los gránulos corticales con formaciones PAS positivas y menciona que la aparición de éstas se produce en forma simultánea

En *Rana pipiens*, *Xenopus laevis* y *Bufo arenarum*, los gránulos corticales se observan por primera vez en el cortex del oocito, durante el estadio de vitelogénesis temprana o primaria (WARD & WARD, 1968; DUMONT, 1972; VALDEZ TOLEDO & PISANÓ, 1980) BALINSKY & DAVIS (1963) describieron a los gránulos corticales como cuerpos con matriz granular de aspecto homogéneo, de 2 μm de diámetro que aparecen durante la vitelogénesis primaria. En *M. stelzneri*, se evidencian durante la etapa de vitelogénesis activa, a la vez que se observa un aumento de los complejos de Golgi y de las vesículas de vitelo. Las observaciones con microscopio electrónico de transmisión mostraron similitudes en cuanto al aspecto de la sustancia que compone los gránulos corticales pero, con respecto al tamaño de los mismos, no se hallaron coincidencias respecto de las especies aludidas (KEMP & ISTOK, 1967; ANDREUCCETTI & CAMPANELLA, 1980), siendo los gránulos corticales de *M. stelzneri* los más desarrollados.

Si bien la actividad ovárica de las hembras adultas de *X. laevis* y *B. arenarum* no son equivalentes (CALLEN et al., 1986; ECHEVERRÍA & GONZALEZ, 1994), se debe destacar que *M stelzneri* comparte, con las especies mencionadas, los patrones generales del ciclo de la vitelogénesis. En los oocitos de *M. stelzneri*, una banda de *material basófilo perinuclear* se hace evidente al final de la previtelogénesis y en los oocitos en vitelogénesis temprana, y un *halo subnuclear* de características basófilas se halla en los oocitos maduros. Una banda discontinua de material citoplasmático similar a la hallada en *M. stelzneri* ha sido puesta de manifiesto en *Bufo lentiginosus* (KING, 1908) y en *B. arenarum* (ECHEVERRÍA, 1974, 1987) con técnicas histológicas generales y reacciones histoquímicas para condrioma. TOURTE et al. (1984) confirman por primera vez en *X. laevis* la presencia de mitocondrias en el citoplasma agrupadas en una *corona perinuclear*. Cabe señalar que *X. laevis* y *M. stelzneri* presentan mitocondrias agrupadas por fuera de la membrana nuclear que se denominan *corona* ("crown", TOURTE et al., 1984) y *halo subnuclear* respectivamente. El halo subnuclear contiene, además, gránulos de glucógeno que podrían motivar la respuesta positiva a la reacción de PAS, mientras que las zonas basófilas del citoplasma de *M. stelzneri*, análogas a la corona perinuclear de *X. laevis*, no reaccionan con PAS.

Tanto el número como la morfología de los nucléolos de los oocitos de *M. stelzneri* presentan variaciones en el transcurso del desarrollo. En oocitos muy jóvenes y en los maduros, los nucléolos presentan una forma esferoidal, pero en estadios intermedios, éstos pueden adoptar distintas morfologías: bipartitos, anulares y vacuolados. Se han descrito nucléolos en forma de collar y de anillo en diferentes especies de anfibios como *Plethodon cinereus* (MACGREGOR, 1965), *Ambystoma mexicanum* (CALLAN, 1966) y *Notophthalmus viridescens* (LANE, 1967), siendo poco frecuente la descripción de estos nucléolos en los anuros En *Bufo arenarum*, ECHEVERRÍA (1980) los describe con una morfología semejante a los que hemos hallado en *M. stelzneri*. LANE (1967) y VAN GANSEN & SCHRAM (1972) propusieron que la variación morfológica aludida es producida por cambios en la

distribución en el ADN y por la segregación de los constituyentes granulares y fibrilares del nucléolo respectivamente. Los *nucléolos bipartitos* hallados en *M. stelzneri* son equivalentes a los nucléolos vacuolares descritos en *B. arenarum* por ECHEVERRÍA (1980). Se utilizó el término bipartito debido a que presentan por lo menos dos partes con distinta afinidad por los colorantes básicos, a fin de distinguirlos de los nucléolos vacuolados hallados en *X. laevis* por DUMONT (1972) y en *M. stelzneri*, que contienen areas de material con menor afinidad por los colorantes básicos.

Resumen

En hembras maduras de *Melanophryniscus stelzneri* (N = 17) de las Sierras de Córdoba (Tanti) y de San Luis (El Trapiche), se identifican cinco estadios de la oogénesis con ocho subestadios (subtipos) estructurados sobre la base de la aparición de vesículas de vitelo, plaquetas vitelinas, gránulos corticales y de pigmento, variaciones morfológicas de la membrana nuclear y de los nucléolos.

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